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(54) Title: NEMATODE-RESPONSIVE PLANT PROMOTERS

(57) Abstract

Nematode-responsive plant promoters, particularly useful in the production of transgenic plants which can produce fixed feeding site cells that become capable of killing, disabling or repelling nematodes or that are themselves killed or rendered unsuitable for nematodes to feed upon when nematodes infect the plants.

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NEMATODE-RESPONSIVE PLANT PROMOTERS

This invention relates to nematode-responsive promoters which can be isolated from plants infected by nematodes and which can either induce (i.e., stimulate) or repress the expression of genes or DNA fragments, under their control, at least substantially selectively in specific cells (e.g., fixed feeding site, pericycle, endodermis, cortex or vascular cells) of the plants' roots, preferably in cells of the plants' fixed feeding sites, in response to the nematode infection. The nematode-induced promoters of this invention are especially useful in transgenic plants for controlling foreign DNAs that are to be expressed selectively in the specific root cells of the plants, so as to render the plants resistant to nematodes, particularly to sedentary endoparisitic nematodes.

This invention also relates to a first or nematode-induced chimaeric gene that can be used to transform a cell of a plant and that contains a first foreign gene or DNA fragment that: a) encodes a product which, when expressed in specific cells of the plant's roots, preferably in cells of fixed feeding sites of the plant, can either kill or at least disturb significantly the specific root cells of the plant, preferably the cells of the plant's fixed feeding sites, or kill, disable or repel nematodes feeding at fixed feeding sites; and b) is under the control of a nematode-induced promoter of this invention.

This invention further relates to a cell of a plant, the genome of which is transformed to contain the first chimaeric gene and optionally a second or restorer chimaeric gene that contains a second promoter controlling

a second foreign gene or DNA fragment encoding a product that is expressed so as to inhibit or inactivate the first foreign gene or DNA fragment or the product encoded thereby in cells other than the specific cells of the plant's roots, preferably in cells other than fixed feeding site cells of the plant.

This invention yet further relates to: a) a nematode-resistant plant (such as tomato or potato) which is regenerated from the plant cell of this invention and is transformed with the first and optionally the second chimaeric genes, b) nematode-resistant plants derived from the regenerated plant and seeds of such plants, and c) plant cell cultures, all of which consist essentially of the transformed plant cells of this invention. The plants of this invention are characterized by the nematode-induced expression of the first chimaeric gene of this invention in their specific root cells, preferably their fixed feeding site cells, and either a) the substantial, preferably complete, absence of expression of the first chimaeric gene in all other plant cells or b) the substantial absence and preferably the complete absence, by expression of the second chimaeric gene of this invention, of the effects of any expression of the first chimaeric gene in all other plant cells -- thereby rendering the plants resistant to nematode infections.

This invention still further relates to a process of rendering a plant resistant to plant-parasitic nematodes by transforming the plant with the first and optionally the second chimaeric gene(s) of this invention.

Background of the Invention

Plant-parasitic nematodes are small (generally 100-300 μm long but up to 4 mm long, and 15-35 μm wide) worm-like animals which feed on root, stem or leaf tissues of living plants. Nematodes are present wherever plants are cultivated. Ectoparasitic nematodes, such as the dagger (Xiphinema and Longidorus spp.), stubby-root (Trichodorus and Paratrichodorus spp.) and spiral (Scutellonema and Helicotylenchus spp.) nematodes, live outside the plant and pierce the plant cells with their stylet in order to feed. Migratory endoparasitic nematodes, such as the lesion (Pratylenchus spp.), stem and bulb (Ditylenchus spp.) and burrowing (Radopholus spp.) nematodes, live and feed inside the plant, migrating through the plant tissues. Sedentary endoparasitic nematodes, such as the root-knot (Meloidogyne spp.), cyst (Globodera and Heterodera spp.), citrus (Tylenchulus spp.) and reniform (Rotylenchulus spp.) nematodes, live and feed inside the plant, inducing specialized fixed feeding sites called giant cells, syncytia or nurse cells in susceptible plants. Such fixed feeding sites serve as food transfer cells for the various developmental stages of the nematodes. Syncytia originate in the pericycle, endodermis or adjacent cortex (Jones, 1981).

Parasitic nematodes can cause significant plant yield losses. The most striking effect of nematode infection is a general reduction in plant growth. Nematodes can act directly as plant pathogens that predispose plants to bacterial or fungal infections or as vectors of plant viruses. Plant diseases caused by nematodes include root galling, root lesions, root rot, stubby roots, stunting and

wilting. Overall average annual yield loss of the world's major crops due to damage by plant-parasitic nematodes is estimated at 12.3 % (Sasser and Freckman, 1987). Monetary losses, when all crops are considered, exceed US $ 100 billion annually (1984 production figures and prices; Sasser & Freckman, 1987).

On a worldwide basis, the ten most significant nematode genera are the ectoparasitic Xiphinema spp., the migratory endoparasitic Pratylenchus spp., Ditylenchus spp., Radopholus spp. and Helicotylenchus spp., the sedentary endoparasitic Meloidogyne spp., Heterodera spp., Globodera spp., Tylenchulus spp. and Rotylenchulus spp. (Sasser and Freckman, 1987). Especially significant are the sedentary endoparasitic nematodes, comprising the genera Heterodera, Globodera and Meloidogyne which cause severe damage to many crops and are of major economic importance. For example, cyst nematodes (e.g., Heterodera and Globodera) cause great problems in the production of potatoes, soybeans, sugar beets, and wheat. Once cyst nematodes have infested a field, it is practically impossible to eliminate them. Meloidogyne spp. affect many species of plants (over 2,000), including most of the major crops of the world. In the tropics, root-knot is often the limiting factor in crop production. In contrast to cyst nematode infections, extensive gall formation accompanies the infection with root-knot nematodes.

Various methods have been used to control plant parasitic nematodes (Brown and Kerry, 1987). They include quarantine measures, manipulation of planting and harvesting dates, improved fertilization and irrigation programs that lessen plant stresses, crop rotation and

fallowing, use of resistant and tolerant cultivars and rootstocks, organic soil amendments, and physical (e.g., solarization), biological and chemical control. Although quarantines are useful, especially when an infestation is first discovered, they are very expensive measures and usually cannot prevent the spread of nematodes (Dropkin, 1980). Furthermore, biological control is difficult to manage, and high quantities and repeated additions of agents are required.

Today, control of plant-parasitic nematodes relies mainly on chemical control. Nematicides used commercially are generally either fumigants (e.g., halogenated aliphatic hydrocarbons and methyl isothiocyanate precursor compounds) or non-fumigants (e.g., organophosphates and oxime-carbamates). However, the use of chemical nematicides poses an increasing number of difficulties:

A. Developing a new nematicide requires a high financial investment, and very few new nematicidal compounds have been discovered despite intensive screening efforts (Morton, 1987). As a result, only a limited number of nematicides are currently available, and new ones are increasingly expensive.

B. Nematocides are only efficient under certain agronomical and environmental conditions (Bunt, 1987), and soil and roots act as barriers, protecting plant-parasitic nematodes from nematicides.

C. Nematodes are known to rapidly invade fields, and phytonematodes are easily distributed with soil and plants. As a result, nematode control with nematicides does not persist for long but must be

repeated frequently and with relatively high concentrations of nematicides to keep nematode populations at low levels.

D. All nematicides are highly toxic. They are therefore hazardous not only to the user but also to the environment. In the USA, several nematicides (e.g., DBCP, EDB, D-D, aldicarb and carbofuran) have already been found in the groundwater (Thomason, 1987). Due to their harmful effects on humans and non-target organisms, their persistence in the soil, and their concentration in ground water, nematicides are being withdrawn from the market worldwide. As a result, there is today a real need to have new, more effective, and safe means to control plant-parasitic nematodes.

In susceptible plants, the infective second-stage juveniles of the sedentary endoparasitic nematode species invade the roots and migrate inter- and intra-cellularly through the cortex to specific regions, usually close to the pericycle, within the roots. Once the nematode has traversed the cortex, it can initiate feeding in a cortical cell, but in most cases, feeding is initiated in endodermal or pericyclic cells (Endo, 1987). Here, the juveniles settle and begin to puncture the cells surrounding heads. Then, the juveniles introduce their secretions into these cells which induces changes in the cells, thereby forming fixed feeding sites in which a large volume of cytoplasm is available to the juveniles (Endo, 1987). The growth cycle of fixed feeding sites is directly related to the further development of the nematodes. After establishment of fixed

feeding sites, the second-stage juveniles increase steadily in size and undergo three moults in quick succession. The third- and fourth-stage juveniles cannot feed, but the young females resume feeding. Thus, further changes in the cells of the fixed feeding sites are controlled by secretions produced by the females and maintained by removal of cytoplasm by the feeding females. The initiation, development and maintenance of fixed feeding sites is essential for the establishment, development and reproduction of the nematodes. The fixed feeding sites are thus critical for the survival of the sedentary endoparasitic nematodes. Without the fixed feeding sites, the nematodes would be unable to feed and reproduce and would die. The sedentary endoparasitic nematodes thus illustrate an important principle -- that the relationship between parasite and plant host depends on a continuing exchange of information by the two organisms. When the nematodes are killed, fixed feeding sites degenerate, leading to the conclusion that the maintenance of fixed feeding sites depends on the continued presence of a functional parasite. Each of the sedentary phytonematodes induces the development of fixed feeding sites upon which it feeds.

Summary of the Invention

In accordance with this invention are provided nematode-responsive cDNA sequences isolated from tomato plants comprising the sequences, SEQ ID no. 1, SEQ ID no. 2, SEQ ID no. 3, SEQ ID no. 4, SEQ ID no. 5, SEQ ID no. 6, SEQ ID no. 7 and SEQ ID no. 8 described in the Sequence Listing.

Also in accordance with this invention are provided nematode-responsive promoters of the tomato genes corresponding to the cDNA sequences of SEQ ID nos. 1-8, particularly:

a) nematode-induced promoters of the genes corresponding to the cDNA sequences of SEQ ID nos. 2-8, more particularly of: i) the gene corresponding to the cDNA sequence of SEQ ID no. 7 which gene is substantially selectively expressed in fixed feeding site cells, particularly in cells within galls, and ii) the genes corresponding to the cDNA sequences of SEQ ID nos. 4 and 6 which genes are substantially selectively expressed in pericycle cells; and

b) a nematode-repressed promoter of the gene corresponding to the cDNA sequence of SEQ ID no. 1.

Further in accordance with this invention is provided the first or nematode-induced chimaeric gene that comprises the following, operably linked, DNA sequences:

1) a nematode-induced promoter that is suitable to direct transcription of a foreign DNA at least substantially selectively, preferably selectively, in the specific root cells, preferably in the cells of fixed feeding sites, of a plant (at whose fixed feeding sites nematodes would feed);

2) a first foreign DNA that encodes a first RNA and/or protein or polypeptide which, when produced or overproduced in the specific root cells, preferably the cells of the fixed feeding sites, of the plant, either a) kills, disables or repels the nematodes when the nematodes feed from the fixed feeding sites or b)

kills the specific root cells, preferably the cells of the fixed feeding sites, or at least disturbs significantly their metabolism, functioning and/or development, thereby at least disturbing significantly, and preferably ending, the ability of the nematodes to feed from the fixed feeding sites of the plant; and

3) suitable 3′ transcription termination signals (i.e., 3′end) for expressing the first foreign DNA in the cells of the specific root cells, preferably the fixed feeding sites.

Still further in accordance with this invention is provided a cell of a plant, in which the nuclear genome is transformed to contain the first chimaeric gene of this invention and preferably, when the nematode-induced promoter directs transcription of the first foreign DNA only substantially selectively in the specific root cells, preferably the fixed feeding site cells, of the plant, to also contain the second or restorer chimaeric gene, preferably in the same genetic locus; the second chimaeric gene comprises the following, operably linked, DNA sequences:

1) a second promoter, such as a nematode-repressed promoter, which can direct transcription of a foreign DNA in cells of the plant where the first foreign DNA is expressed, preferably at least substantially selectively in cells other than the specific root cells, particularly in cells other than the fixed feeding site cells, of the plant;

2) a second foreign DNA that encodes a second RNA and/or protein or polypeptide which, when produced

or overproduced in cells of the plant, inhibits or inactivates the first foreign DNA or the first RNA or protein or polypeptide; and

3) suitable 3' transcription termination signals for expressing the second foreign DNA in cells of the plant.

Still further in accordance with this invention are provided the nematode-resistant plant regenerated from the transformed plant cell of this invention, nematode-resistant plants derived therefrom and their seeds, and plant cell cultures, each of which consists essentially of the plant cells of this invention.

Yet further in accordance with this invention is provided a process for rendering a plant resistant to nematodes, particularly sedentary endoparisitic nematodes, comprising the step of transforming the plant's nuclear genome with the first chimaeric gene and optionally the second chimaeric gene of this invention.

Detailed Description of the Invention

Throughout this Description, the following definitions apply:

"Fixed feeding sites" should be understood as specialized feeding sites (such as giant cells, syncytia and nurse cells and, if present, galls), the formation of which is induced by sedentary endoparasitic nematodes in susceptible plants. The plant cells of such sites serve as food transfer cells for the various developmental stages of the nematodes.

"Nematode-infected plant" means a plant in which a nematode has entered.

"Giant cells" should be understood as the multinucleate plant root cells induced by nematodes such as root-knot nematodes. The multinucleate condition of each giant cell is believed to result from multiple mitosis in the absence of cytokinesis.

"Syncytium" refers to multinucleate plant root cells induced by nematodes such as cyst nematodes. The multinucleate condition of each syncytium results from cell wall dissolution between contiguous cells with preexisting nuclei.

"Nurse cells" refers to a group of six to ten uninucleated plant root cells, induced by Tylenchulus spp., which have a dense cytoplasm without a vacuole and a much enlarged nucleus and nucleolus.

"Galls" refer to a proliferation of cortical plant cells/tissue induced by nematodes. Typically, giant cells reside within galls.

"Nematode-responsive promoter" means a promoter whose action in controlling transcription of a DNA sequence (e.g., gene) in a plant is influenced -- that is, either induced (i.e., stimulated) or repressed -- by infection of the plant by nematodes and preferably is influenced selectively in specific cells of the plant's roots, particularly in cells of the plant's fixed feeding sites. A "nematode-responsive promoter"

can be either a "nematode-induced promoter" or a "nematode-repressed promoter".

"Specific cells of a plant's roots" or "specific root cells of a plant" means cells of a root tissue such as the fixed feeding sites, the pericycle, the endodermis, the cortex or the vascular tissue, preferably a) cells of the fixed feeding sites or b) cells of tissue (e.g., pericycle cells) which i) will differentiate into fixed feeding site cells upon infection of the plant by nematodes or ii) can be altered to reduce the ability of nematodes to feed at fixed feeding sites of the plant. Particularly preferred specific root cells of a plant are fixed feeding site cells.

"Homologous" refers to proteins or nucleic acids having similar sequences of amino acids or nucleotides, respectively, and thus having substantially the same structural and/or functional properties.

"Expression" means transcription and translation to a product from a DNA encoding the product.

"Foreign" with regard to a DNA sequence, such as a first or second foreign DNA of this invention, means that such a DNA is not in the same genomic environment (e.g., not operably linked to the same promoter and/or 3' end) in a plant cell, transformed with such a DNA in accordance with this invention, as is such a DNA when it is naturally found in a

cell of the plant, bacteria, animal, fungus, virus, or the like, from which such a DNA originates.

In accordance with this invention, a nematode-resistant plant can be produced from a single cell of a plant by transforming the plant cell in a known manner to stably insert, into its nuclear genome, the first chimaeric gene of this invention which comprises at least one first foreign DNA that is: under the control of, and fused in frame at its upstream (i.e., 5′) end to, one of the nematode-induced promoters of this invention; and fused at its downstream (i.e., 3′) end to suitable transcription termination (or regulation) signals, including a polyadenylation signal. Thereby, the first RNA and/or protein or polypeptide is produced or overproduced at least predominantly, preferably exclusively, in the specific root cells, preferably cells of the fixed feeding sites, of the plant. Optionally, the plant cell genome can also be stably transformed with the second chimaeric gene, comprising at least one second foreign DNA that is: under the control of, and is fused at its 5′ end to, the second promoter which is capable of directing expression of the second foreign DNA in cells of the plant where the first foreign DNA is expressed, preferably substantially selectively in plant cells other than the specific root cells, particularly in cells other than the fixed feeding site cells; and fused at its 3′end to suitable transcription termination signals, including a polyadenylation signal. The second chimaeric gene is preferably in the same genetic locus as the first chimaeric gene, so as to guarantee, with a high degree of certainty, the joint segregation of both the first and

second chimaeric genes into offspring of the plant regnerated from the transformed plant cell. However in some cases, such joint segregation is not desirable, and the second chimaeric gene should be in a different genetic locus from the first chimaeric gene.

In accordance with this invention, the first foreign DNA, controlled by the nematode-induced promoter, encodes a first RNA and/or protein or polypeptide which, when produced or over-produced in the specific root cells, preferably the cells of the fixed feeding sites, of the plant, either: a) kills such cells or at least disturbs significantly their metabolism, functioning and/or development so as to at least disturb significantly, and preferably end, the ability of nematodes to feed from the fixed feeding sites; and/or b) kills, disables or repels any nematode(s) feeding at the fixed feeding sites. First foreign DNAs preferably encode, for example, the following which can kill the specific root cells, preferably fixed feeding site cells, or at least disturb significantly their metabolism, functioning and/or development: RNases such as RNase T1 or barnase; DNases such as endonucleases (e.g. EcoRI); proteases such as papain; enzymes which catalyze the synthesis of phytohormones, such as isopentenyl transferase or the gene products of gene 1 and gene 2 of the T-DNA of Agrobacterium; glucanases; lipases; lipid peroxidases; plant cell wall inhibitors; or toxins such as the A-fragment of diphtheria toxin or botulin. Other preferred examples of such first foreign DNAs are antisense DNAs complementary to genes encoding products essential for the metabolism, functioning and/or development of the specific root cells, preferably the fixed feeding site

cells. First foreign DNAs preferably encode, for example, the following first polypeptides or proteins which can kill or disable nematodes: the Bacillus thuringiensis toxins described in European patent publication ("EP") 303426 (which is incorporated herein by reference), collagenases, chitinases, glucanases, peroxidases, superoxide dismutases, lectins, glycosidases, antibacterial peptides (e.g., magainins, cecropins and apidaecins), gelatinases, enzyme inhibitors or neurotoxins. When the nematode-induced promoter is a pericycle-specific promoter, such as the promoter of the gene corresponding to the cDNA of SEQ ID no. 4 or 6, the first foreign DNA under the control of such a promoter preferably encodes either: a) a material such as callose or lignin which, when produced in the pericycle cells, will make the pericycle substantially impenetrable to nematodes, so as to prevent the nematodes from feeding at the fixed feeding sites or establishing other fixed feeding sites and thereby repel the nematodes from the fixed feeding sites. Plants transformed with such a first foreign DNA in a first chimaeric gene of this invention will be resistant to nematode infections either because of a nematode-induced breakdown of their fixed feeding sites, which are essential for the survival of nematodes, or because nematodes, feeding on the fixed feeding sites, will be killed, repelled or disabled by, for example, a nematode toxin produced in situ by their fixed feeding site cells.

Each of the nematode-induced promoters of this invention, particularly the promoter of the gene corresponding to the cDNA of SEQ ID no. 7, which can be used to control expression of the first foreign DNA of this invention substantially exclusively, preferably

exclusively, in the specific root cells, particularly fixed feeding site cells, of a plant, and each of the nematode-repressed promoters of this invention, which can be used to control expression of the second foreign DNA of this invention predominantly, preferably substantially exclusively, in cells other than the specific root cells, particularly cells other than the fixed feeding site cells of a plant, can be identified and isolated in a well known manner in the specific root cells, particularly the fixed feeding site cells, of the plant. For example, a suitable nematode-induced or nematode-repressed promoter can be identified and isolated in one or more plants, preferably two or more plants (e.g., tomato and potato), infected with nematodes by the following process steps:

1. searching for an mRNA which is, respectively, substantially present or substantially absent in the cells of the roots of the plant(s) after nematode infection thereof by construction of a cDNA library and differential screening;
2. isolating the cDNA that corresponds to the nematode-responsive mRNA;
3. using these cDNA as a probe to identify the regions in the plant(s) genome(s) which contain DNA coding for the nematode-responsive mRNA; and then
4. identifying the portion of the plant genome(s) that is upstream (i.e., 5') from this DNA and that codes for the nematode-responsive promoter of this DNA.

The nematode-responsive cDNA clones of step 3 of this process can also be isolated by other methods (Hodge et al, 1990). Examples of nematode-responsive promoters, which

can be obtained by this process, are the promoters of this invention which can be identified using the cDNAs of SEQ ID nos. 1-8, particularly the nematode-induced promoters which can be identified with the cDNAs of SEQ ID nos. 2-8 and the nematode-repressed promoter which can be identified with the cDNA of SEQ ID no.1. Certain of the nematode-induced promoters of this inention, such as that which can be identified with the cDNA sequence of SEQ ID no. 6, causes expression of the first chimaeric gene in all cells of a nematode-infected plant, transformed with the first chimaeric gene, but is believed to cause expression at substantially higher levels in fixed feeding site cells. For this reason, at least certain of the nematode-induced promoters are preferably combined in the first chimaeric gene with a first foreign DNA selected so that its differential expression in the specific root cells, particularly fixed feeding site cells (as compared to the other cells of the infected plant), has the desired selective effect on the specific root cells, preferably the fixed feeding site cells. Other promoters of this invention, such as those which can be identified by means of the cDNA sequences of SEQ ID no. 4 and SEQ ID no. 6, cause expression of the first foreign DNA predominantly in pericycle cells.

When the nematode-induced promoter in the first chimaeric gene of this invention is not 100% specific for the specific root cells, preferably the fixed feeding site cells, of a plant transformed therewith, it is preferred that the plant be further transformed so that its nuclear genome contains, stably integrated therein, the second chimaeric gene of this invention. The second promoter of

the second chimaeric gene is selected so that it is capable of directing transcription of the second foreign DNA to provide sufficiently high expression levels of the second RNA or protein or polypeptide to inhibit or preferably inactivate the first RNA or protein or polypeptide in all plant cells, with the exception of the specific root cells, preferably the fixed feeding site cells. An example of the second promoter is a nematode-repressed promoter of this invention, such as the promoter of the gene which can be identified with the cDNA of SEQ ID no. 1. Other examples of second promoters are: the strong constitutive 35S promoters of the cauliflower mosaic virus of isolates CM 1841 (Gardner et al, 1981), CabbB-S (Franck et al, 1980) and CabbB-JI (Hull and Howell, 1987); and the TR1′ and TR2′ promoters which drive the expression of the 1′ and 2′ genes, respectively, of the T-DNA (Velten et al, 1984). Alternatively, a second promoter can be utilized which is specific for one or more plant tissues or organs, such as roots, whereby the second chimaeric gene is expressed only in cells of the specific tissue(s) or organ(s). Another alternative is to use a promoter whose expression is inducible (e.g., by temperature or chemical factors). To control root-knot nematodes, it may be preferred that the second chimaeric gene be under the control of a gall-specific promoter.

In accordance with this invention, the second foreign DNA, controlled by the second promoter, encodes a second RNA and/or protein or polypeptide which, when produced or overproduced in cells of a plant, inhibits or preferably inactivates the first RNA, protein or polypeptide in such cells. Second foreign DNAs preferably encode, for example,

the following: barstar which neutralizes the activity of barnase (which degrades RNA molecules by hydrolyzing the bond after any guanine residue); EcoRI methylase which would prevent the activity of the endonuclease EcoRI; or a protease inhibitor which would neutralize the activity of a protease, such as papain (e.g., papain zymogen and papain active protein). Another preferred example of a second foreign DNA is a DNA which encodes a strand of antisense RNA which would be complementary to a strand of sense first RNA.

In the first and second chimaeric genes of this invention, 3' transcription termination signals or 3'ends can be selected from among those which are capable of providing correct transcription termination and polyadenylation of mRNA in plant cells. The transcription termination signals can be the natural ones of the first and second foreign DNAs, to be transcribed, or can be foreign. Examples of foreign 3' transcription termination signals are those of the octopine synthase gene (Gielen et al, 1984) and of the T-DNA gene 7 (Velten and Schell, 1985).

The cell of a plant, particularly a plant capable of being infected with Agrobacterium, can be transformed using a vector that is a disarmed Ti-plasmid containing the first chimaeric gene and optionally the second chimaeric gene of this invention and carried by Agrobacterium. This transformation can be carried out using the procedures described, for example, in EP 116,718 (29 August 1984), EP 270,822 (15 June 1988) and Gould et al (1991) [which are also incorporated herein by reference]. Preferred Ti-plasmid vectors contain the foreign DNA sequences between

the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 233,247), pollen-mediated transformation (as described, for example, in EP 270,356, PCT publication WO 85/01856, and US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 67,553 and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475). In case the plant to be transformed is corn, rice or another monocot, it is preferred that more recently developed methods be used such as, for example, the methods described for certain lines of corn by Fromm et al (1990) and Gordon-Kamm et al (1990), the methods described for rice by Datta et al (1990) and Shimamoto et al (1989) and the more recently described method for transforming monocots generally of PCT patent application no. PCT/EP 9102198.

The first and second chimaeric genes of this invention are preferably inserted in the same genetic locus in the plant genome. Therefore, it is preferred that the first and second chimaeric genes be transferred to the plant genome as a single piece of DNA, so as to lead to their insertion in a single locus in the genome of the plant. However, plants containing the two chimaeric genes can also be obtained in the following ways:

1. The chimaeric genes can be separately transferred to the nuclear genomes of separate plants in independent transformation events and can subsequently be combined in a single plant genome through crosses.

2. The chimaeric genes can be separately transferred to the genome of a single plant in the same transformation procedure, leading to the insertion of the respective chimaeric genes at multiple loci (cotransformation).

3. One of the two chimaeric genes can be transferred to the genome of a plant already transformed with the other chimaeric gene.

The resulting transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the first chimaeric gene and optionally the second chimaeric gene in other varieties of the same or related plant species. Seeds obtained from the transformed plants contain the chimaeric gene(s) of this invention as a stable genomic insert.

The Examples, which follow, describe the isolation and characterization of nematode-responsive cDNA sequences of this invention of SEQ ID nos. 1-8 and their use as molecular probes for isolating and identifying the corresponding genomic sequences. Once the corresponding genomic sequences have been identified, the promoter regions are isolated according to well-known methods as described, for example, in European patent applications ("EPA") 89401194.9 and 90402281.1.

Unless stated otherwise in the Examples, all nucleic acid manipulations are done by the standard procedures described in Sambrook et al, Molecular Cloning : A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, N.Y. (1989). Oligonucleotides are

designed according to the general rules outlined by Kramer and Fritz (1988) and synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981) on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarssen, Netherlands).

In the following Examples, reference is made to the following Sequence Listing (SEQ ID nos. 1-8) :

SEQUENCE LISTING

SEQ ID no. 1 : LEMMI 1 cDNA (Between brackets)
SEQ ID no. 2 : LEMMI 2 cDNA (Between brackets)
SEQ ID no. 3 : LEMMI 4 cDNA (Between brackets)
SEQ ID no. 4 : LEMMI 7 cDNA (Between brackets)
SEQ ID no. 5 : LEMMI 8 cDNA (Between brackets)
SEQ ID no. 6 : LEMMI 9 cDNA (Between brackets)
SEQ ID no. 7 : LEMMI 10 cDNA (Between brackets)
SEQ ID no. 8 : LEMMI 11 cDNA (Between brackets)

Example 1 : ISOLATION AND CHARACTERIZATION OF NEMATODE-RESPONSIVE cDNAs FROM TOMATO

Young tomato plants (Lycopersicon esculentum cv. Marmande) were each grown at 20° C in industrial pots under semi-sterile conditions in a 1:1 sand:soil mixture, which was sterilized by irradiation and watered daily with a filtered sterilized nutrient solution (Cooper, 1976). Plants were infected by inoculation with about 6,000 Meloidogyne incognita race 1 eggs per pot. The nematode inoculum was obtained as described by Hussey and Barker (1973). Infected and control plants were grown under identical conditions. Five weeks after inoculation, plant material was harvested from both infected and control plants, frozen under liquid nitrogen and stored at - 80°C for further processing. Total RNA was prepared from frozen

tissue (-70° C) according to Jones et al (1985). Poly $(A)^+$RNA was isolated by oligo - dT cellulose affinity chromatography as described by Slater (1984).

In order to construct a cDNA library from infected tomato plants, mRNA was extracted from hundreds of Meloidogyne incognita race 1 induced root-knots. cDNA was synthesized using the cDNA synthesis system plus (Amersham Intl. PLC, Buckinghamshire, England). The cDNA library was constructed in plasmid pUC19 (Yanisch-Perron et al, 1985) which was electroporated in E. coli. About 3,000 randomly selected clones were individually grown in the wells of microtiter plates containing LB medium (Miller, 1972) supplemented with 100 µg/ml ampicillin. Replicas of the sublibrary were made with a replica block on Hybond N nylon membranes (Amersham) which were further treated according to Sambrook et al (1989).

First strand cDNAs, reverse transcribed from total RNA of root-knots and of control roots, were used as probes for differential screening. To this end, reproducible replicas of 3,000 individual cDNA clones were hybridized overnight at 68°C with ^{32}P-labeled probes. Subsequently, the hybridization patterns obtained with cDNA probes from root-knots were compared to those obtained with cDNA probes from control roots. Ninety-three (93) clones gave a stronger hybridization signal with the "infected" probes than with the "control" probes. These clones were labeled as "nematode-stimulated" or "nematode-induced" clones and were subjected to a second screening. Several clones gave a weaker hybridization signal with the "infected" probes than with the "control" probe, and one of these clones was

labeled as a "nematode-repressed" clone and subjected to a second screening.

The one "nematode-repressed" and eight (8) of the "nematode-stimulated" LEMMI (Lycopersicon esculentum cv. Marmande - Meloidogyne incognita race 1) cDNA clones showed pronounced differential hybridization patterns and were selected for further analysis. The following cDNA clones showed a nematode-stimulated hybridization pattern: LEMMI 2, LEMMI 4, LEMMI 6, LEMMI 7, LEMMI 8, LEMMI 9, LEMMI 10 and LEMMI 11. The following cDNA clone showed a nematode-repressed hybridization pattern: LEMMI 1. Cross-hybridization performed under high stringency conditions showed that LEMMI 6 and LEMMI 9 most likely correspond to the same mRNA.

The different cDNA clones were sequenced. According to a database search, LEMMI 8 and LEMMI 11 appeared to be an extensin. Southern blot analysis of tomato DNA performed under high stringency conditions proved the plant origin of these clones. Since root-knots contain nematodes, some of the nematode-stimulated clones could have been from nematode origin. Southern blot and northern blot analysis further showed that LEMMI 4 and LEMMI 8 belong to different multigene families. The differential hybridization patterns of the cDNA clones were confirmed by Northern blot analysis. In situ hybridization experiments on tissue sections of nematode-infected in vitro grown tomato plants using LEMMI 7, LEMMI 9 and LEMMI 10 as probes showed that: both LEMMI 7 and LEMMI 9 are predominantly expressed in pericycle cells and LEMMI 10 has high specificity for fixed feeding site cells.

Example 2 : ISOLATION AND CHARACTERIZATION OF NEMATODE-RESPONSIVE cDNAs FROM POTATO

Potato plants (Solanum tuberosum cv Bintje) were infected by inoculation with the potato cyst nematode, Globodera pallida. Infected and control plants were grown under identical conditions. Eight weeks after inoculation, infected roots were harvested, and RNA was prepared as described in Example 1. 5 μg of poly $(A)^+$RNA was used as a starting material for the construction of a cDNA library. A cDNA library of 40,000 recombinant clones was obtained after ligation in the plasmid vector pUC18 (Norrander et al, 1983) and electroporation in E. coli. 3,700 of these clones were isolated and grown in microtiter wells. Subsequently, these clones were subjected to a differential screening procedure as described in Example 1 to identify nematode-repressed cDNAs and nematode-stimulated cDNAs of potato.

Example 3: IDENTIFICATION AND CHARACTERIZATION OF OTHER NEMATODE-RESPONSIVE GENES FROM PLANTS

For the purpose of identifying plant genes which are induced by nematodes, tobacco and Meloidogyne javanica were used as a model system. An in vitro system has been developed which allows synchronized infection by a number of nematodes and immediate analysis of resulting proteins. The system used in vitro grown SR1 tobacco plants as a starting material.

Explants consisting of an internode and a leaf were cut off tobacco plants. The explants were then put into Petri dishes (13.5 cm diameter) which contain the normal culture medium used for SR1 tobacco plants. The explants started rooting after about 5 to 7 days. After 10 days, the

roots were infected in the following way: the culture medium is carefully lifted and a solution containing approximately 1000-2000 nematode larvae (2nd larval instar) is added. This in vitro system had several advantages, including the synchronicity of the infection, the easy scoring and the possibility of stage-specific observations. Several pathogen-induced and pathogenesis-related proteins were tested using this system. A very strong induction of extensin (8-fold higher than in control roots) was observed. Subsequently, the promoter of the unique extensin gene of Nicotiana plumbaginifolia (De Loose et al, 1991) was fused to a reporter gene, ß-glucuronidase (Jefferson et al, 1986), and transformed into tobacco. These transformed plants were analysed by means of histochemical ß-glucuronidase assays (Peleman et al, 1989). A very localized and strong Gus-activity was observed around the fixed feeding sites.

EXAMPLE 4 : ISOLATION OF NEMATODE-RESPONSIVE GENES CORRESPONDING TO THE NEMATODE-RESPONSIVE cDNA CLONES OF EXAMPLES 1 AND 2

In order to isolate the genomic DNA clones carrying the regulatory sequences of the genes corresponding to the selected cDNA clones of Examples 1 and 2, a genomic library is constructed. To this end, total genomic DNA of tomato is digested with a tetra-cutter restriction enzyme in order to obtain approximately 20 kb DNA fragments. These genomic DNA fragments are then cloned in the phage vector, Charon 35 (Rimm et al, 1980).

The nematode-responsive cDNAs of Examples 1 and 2 are used as probes for screening the library. Genomic clones, which hybridize to the probes, are selected and sequenced.

Comparison of the sequences from the cDNA clones of this invention with those of the genomic clones leads to the identification of the homologous regions. At the 5′ end of the homologous region of each genomic clone, the ATG translation initiation codon and TATA consensus sequence are identified in order to locate the nematode-responsive promoter region. The fact that the "TATA-box" is part of the promoter region is confirmed by primer extension.

Confirmation of the nematode-responsive promoter regions is made by use of the "inverse PCR" technology as described by Ochman et al (1988, 1989). By this method, the DNA sequences flanking a well-defined core region of each nematode-responsive gene sequence, which corresponds to the sequence of a nematode-responsive cDNA, are amplified.

Example 5 : CONSTRUCTION OF NEMATODE-RESPONSIVE PROMOTER CASSETTES DERIVED FROM THE NEMATODE-RESPONSIVE GENES OF EXAMPLE 4

The 5′ regulatory sequences, including the nematode-responsive promoter, of each of the nematode-responsive genes of Example 4 are subcloned into the polylinker of pMAC 5-8 (EPA 87402348.4). This produces vectors which can be used to isolate single stranded DNA for use in site-directed mutagenesis. Using site-directed mutagenesis (EPA 87402348.4), sequences surrounding the ATG translation initiation codon of the 5′ regulatory sequences of each of the nematode-responsive genes are modified to create a unique recognition site for a restriction enzyme, for which there is a corresponding recognition site at the 5′ end of the first foreign DNA of this invention (that is to be fused to the 5′ regulatory sequences in Example 6, below). The resulting plasmids each contain the newly created

restriction site. The precise nucleotide sequence spanning each newly created restriction site is determined in order to confirm that it only differs from the 5′ regulatory sequences of the corresponding nematode-responsive gene by the substitution, creating the new restriction site.

Example 6 : CONSTRUCTION OF PLANT TRANSFORMATION VECTORS FROM THE PROMOTER CASSETTES OF EXAMPLE 5

Using the procedures described in EPA 89401194.9 and 90402281.2, the promoter cassettes of Example 5 are used to construct plant transformation vectors comprising first chimaeric genes of this invention, each of which contains the 5′ regulatory sequences, including the nematode-responsive promoter, of one of the nematode-responsive genes isolated in Example 4. Each of these 5′ regulatory sequences is upstream of, is in the same transcriptional unit as, and controls a first foreign DNA (from EPA 89401194.9) encoding barnase from Bacillus amyloliquefaciens (Hartley and Rogerson, 1972). Downstream of the first foreign DNA is the 3′ end of the octopine synthase gene (Gielen et al, 1984). Each chimaeric gene also comprises the 35 S′3 promoter (Hull and Howell, 1987) fused in frame with the neo gene encoding kanamycin resistance (EPA 84900782.8), as a marker, and the 3′ end of the octopine synthase gene.

Example 7 : TRANSFORMATION OF TOMATO AND POTATO WITH THE PLANT TRANSFORMATION VECTORS OF EXAMPLE 6

To obtain transformation of, and major expression in, tomato and potato by the plant transformation vectors of Example 6, each vector is inserted between the T-DNA border sequences of a Ti-plasmid carried by Agrobacterium (EPA 89401194.9 and EPA 90402281.1). In this regard, the

vectors from Example 6 are each mobilized into Agrobacterium tumefaciens C58C1 Rif^R containing pMP90 (Koncz and Schell, 1986). The resulting recombinant Agrobacterium strains are used to transform tomato leaf discs using the standard procedures described in EPA 87400544.0. The resulting recombined Agrobacterium strains are also used to transform potato plants (Solanum tuberosum cv. Bintje) by means of tuber disc infection as described by Deblock et al (1987). Transformed calli are selected on a substrate containing 100 µg/ml kanamycin, and resistant calli are regenerated into plants.

Plants transformed with the nematode-induced chimaeric genes of this invention containing nematode-induced promoters, particularly the nematode-induced promoters of Example 4 identified with the cDNAs of SEQ ID nos. 2-8, quite particularly the promoter identified with the cDNA of SEQ ID no. 7, show a significantly higher degree of resistance to sedentary endoparasitic nematode infection, such as Meloidogyne incognita infection, than do non-transformed control plants. As a result, the transformed plants have significantly lower yield losses than do the control plants.

Needless to say, the use of the nematode-responsive promoters of this invention is not limited to the transformation of any specific plant(s). Such promoters can be useful in transforming any crop, such as rapeseed, alfalfa, corn, cotton, sugar beets, brassica vegetables, tomato, potato, soybeans, wheat or tobacco where the promoters can control gene expression, preferably where such expression is to occur abundantly in specific root cells, preferably in fixed feeding site cells.

Also, the use of the nematode-responsive promoters of this invention is not limited to the control of particular foreign DNAs but can be used to control expression of any gene or DNA fragment in a plant.

Furthermore, this invention is not limited to the specific nematode-responsive, preferably nematode-induced, promoters described in the foregoing Examples. Rather, this invention encompasses promoters equivalent to those of the Examples which can be used to control the expression of a structural gene, such as a first foreign DNA, at least substantially selectively in specific root cells, preferably fixed feeding site cells, of a plant. Indeed, it is believed that the DNA sequences of the promoters of the Examples can be modified by replacing some of their codons with other codons, provided that such modifications do not alter substantially the ability of polymerase complexes, including transcription activators, of specific root cells, particularly fixed feeding site cells, to recognize the promoters, as modified.

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SEQUENCE LISTING

1. General Information

i) APPLICANT : PLANT GENETIC SYSTEM N.V.

ii) TITLE OF INVENTION : Nematode-Responsive Plant Promoters

iii) NUMBER OF SEQUENCES: 8

iv) CORRESPONDENCE ADDRESS :
A. ADDRESSEE : Plant Genetic Systems N.V.
B. STREET : Plateaustraat 22,
C. POSTAL CODE AND CITY : 9000 Ghent,
D. COUNTRY : Belgium

v) COMPUTER READABLE FORM :
A. MEDIUM TYPE 5.25 inch, double sided, high density 1.2 Mb floppy disk
B. COMPUTER : IBM PC/AT
C. OPERATING SYSTEM : DOS version 3.3
D. SOFTWARE : WordPerfect 5.1

vi) CURRENT APPLICATION DATA : Not Available

(vii) PRIOR APPLICATION DATA : EPA 91401421.2

SEQ ID NO. 1

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 1193 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato

PROPERTIES: Nematode-responsive cDNA 1
MISCELLANEOUS: cDNA desinated as LEMMI 1

```
TGTGNGTTGC TCNCTCNTTG GCNCNCCGGC TTTNCNCTTT NTGCTTCCGG      50
CTCGTNTGTT GTGTGGNNTT GTGTGNGNTT NNCNNTTTCN CNCNGGNNNC     100
GCTATGNCCA TGATTACGCA AGCTTGCATT CCTGCAGGTC GACTCTAGAG     150
GATCCCCGGG TACCGAGCTC GCCATGGTAG GCGGATCCTC GAATTCGAGG     200
ATCCGGGTAC CATGCAGAAA CTAGAACTCA AATACCAAGA GTCAGTTTCT     250
GAAGAATATC AATGGCGTCA CTTCAGTGCA AAAGCCAGTT GCACAACCAA     300
CTGTCTGCCA AAAAACCACC AACACTGTCA CTTGCCACAA GGCGAATGAG     350
CACCACTTGG CTGACAAAAT GAAACAGATG ACAACCAAGA TGTTGCACCA     400
CGACAACCAT GGTCAGCAAT CTGCCTGCCA TGGAGCCAAA ACTCAACATT     450
CAGCAGGCCA TGGCTCCACT GCTATTCACG GAAATCATGG TAGCCACTGC     500
CAGCAGACTG CATGCCATGG TGCAAAAACT CAACATTCAG CATGCCATGG     550
CTCCACTGCT ATGCATGGAA ATCATGCTAA GGGACACAAC ACTGCATGCC     600
ATGATGCTAA ANCTCAACAT TCAGCNGGCC ATGGCTCCAC TGTTGGGCAT     650
GGAAATCATG CCAAGGGCAC AACNCTGCAT GCCATGGMAC CAAAWCTCAA     700
CATTCAACAT GCCATGGTCC ACCGCTACTC ATGGAAAYAT GCTAWKNNNA     750
GATNACTGNA TGCATGGCAN NAAAACTCAA NATTCAANAG GCCATGGCTC     800
CACTGCTATG CATGGAGNTT ATGCTAACCA CGGACAGNAC ACTGCAAGTC     850
ATGGCTCCAC TGCTGTGCAT GGAAGTCATG GTAGCCACAA CCAGCAGACT     900
GTGTCCATGG CTCAAAGAAG GAAGGGGGCA TCATGCATAA GATAGGTAGT     950
CAGCTGAAGA CCATCGGGAA AAAGAAGAAC AAAGATGGAC ACTGCAGAGA    1000
TGGCAGTGAC AGCAGCGACA GCAGCAGCAG CAGCGATGAT GAGAGCGACA    1050
ATGAGAATTG TGGAAAAAAA AAAAACCATG GTACCCGGAT CCTCGAATTC    1100
ACTGGCCGTC GTTTTACAAC TTCGTGACTG GGAAANCCCT NGCGTTACCC    1150
AACTTAATNG CCTTGCNGCA CATCCCCCTT NNNCCAGCTG GGT           1193
```

SEQ ID NO. 2

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 305 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato

PROPERTIES: Nematode-responsive cDNA
MISCELLANOUS: cDNA designated as LEMMI 2

```
GAATGNNNNN NNGGGGNNNN NNAAGNGNNA AAGNNNAAAG GAGAGAAAGA      50
CAAGTCAAAG NGGAGTCAGN AGAAGAGAAG GATATTGNNN AAGNNNAGAA     100
GGATAAAGAG AAGAAAGACA AGAAAAAAGG GGCATCAGAC GAAGAGAACG     150
AGCGCGAAGA AGAGAATGAT GAAAAAGGTG TGAAAAAAAA AAAAAAACCA     200
TGGTACCCGG ATCCTCGAAT CGAGGATCCG NNNNNNATNG CGAGCTCGGT     250
ACCCGGGGAT CCTCTAGAGT CGACCTGCAA ACATGCAAGC TTGGNGTAAT     300
CATGT                                                      305
```

SEQ ID NO 3

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 543 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato

FEATURES: Nucleotide 1 to 64: cloning vector sequence
Nucleotide 65 to 88: cloning adaptor sequence

PROPERTIES: Nematode-responsive cDNA
MISCELLANEOUS: cDNA designated as LEMMI 4

```
ACATGATTAC NCCAAGCTTN CATGCCTGCA GGTCGACTCT AGAGGATCCC      50
CGGGTACGAG CNCNAATTCG AGGATCCGGG TACATGGCTG GTTTTCTACG     100
AAATATATTT TAGTAAAGCA ATTTTTAGAA AAACAAAGAA GTAGAGATAC     150
TCTCCGAGAT AAAGTAATTT CATTAACTCA TCTTTTGAAA CTCACAAAGC     200
TTATGTTTTA ACAAGCATCA GACAGACATA AAGCNCGAAG GTTTTATGTC     250
TAAAGGGAAA CAGCCCAGAT GAATAGATAA GGTCCCAAAT AGTTATTAAA     300
AATAAAATAA GTTTTCATTT AAAAGATTAA AAGGTATGCT TGGAAGCAGC     350
CAGCCTTTAA AGAAAGCGTT GCAGCTCATT AATTGAATAA TAANAACTTA     400
TTGAGAATAT ACGGGAATGA AATAACTCAC AAAAAACCAA CCGAATCTTT     450
CAATATGTTA ATAAAAAGTA TGTCATATGG TAGTAGAATA TNTTGTCANA     500
AAAATAAAAC NAAAGAGAAA ATCTNTTGGT ATAAGTAGCG AAA           543
```

SEQ ID NO.4

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 482 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato

FEATURES: Nucleotide 1 to 8: cloning vector sequence
Nucleotide 9 to 32: cloning adaptor sequence
Nucleotide 33 to 482: putative Open Reading Frame ("ORF")

PROPERTIES: Nematode-responsive cDNA
MISCELLANEOUS: cDNA designated as LEMMI 7

```
GGCCAGTGAA TTCGAGGATC CGGGTACCAT GGAATTATTC TCAACCAATG      50
GGTGAAAAAA TCAAAGTTGA AGGAGCAGAG AAGAAGAACG AAAGTTCAAT     100
TGTTTTAAAA CTGGATTTGC ATTGTGAAGG TTGTGCACAA AAACTCAGAC     150
GATTCATTCG CCATACTCAT GGTGTGGAAA AAGTGAAATC GGATTGTGAA     200
ACTGGAAAAC TGACGGTTAA AGGTGACGTT GACCCTTCAT GGCTCCGGGA     250
GAGAGTGGAG ATCAAAACCA AAAAGAAGGT GGAGCTTATA TCATCGCCGC     300
CCAAAAAGGA CNCCGGAGAT AAAAAGAGCG GCGGAGATAA AAAGTCGGTG     350
AAAAAACAGA GGACAAGAAG GAAGACGAGA AGAAACCCAA AGAGGCTCAA     400
GTAACAGTGT GGTGGCATTA AAGATTCGGG TGCTTGTGAT GGATGTGCAC     450
ATAAAATCAA ACGAGTTATT AAAAAGATTA AA                        482
```

SEQ. ID. NO. 5

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 2610 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato
FEATURES: nucleotides 1878 to 2434 constitute nematode-responsive cDNA
PROPERTIES: Nematode-responsive cDNA
MISCELLANEOUS: cDNA designated as LEMMI 8

```
ATAACAATTT CACACAGGAA ACAGCTATGA CCGATGATTA CGCCAAGCTT      50
GCATGCCTGC AGGTCGACTC TAGAGGATCC CCGGGTACCG AGCTCGAATT     100
CGAGGATCCG GGTACCATGG ACTCCGAAAA TATCAGGAAT AGCAGTGAAT     150
CGAGCGGGAG GGCGAGCGAG TAAGCGAGAT CGGAGATCGG AAGATGTCGT     200
CGGAACCACC GCCATTTCAA GAAGCTTCAC GTTGTGATGT CTGCAATTGC     250
AGCTTCAATA CTTTCCGGCG ACGGCACCAT TGCAGATGTT GCGGCCGAAC     300
ATTATGTGCT GAACATTCAG CAAATCAGAT GGCCTTGCCA CAATTTGGTC     350
TTCACTCAAG TGTGAGAGTT TGTGGAGATT GTTTTAATAA CTCCTCTCGG     400
TAAATTTCTG CCACTATTAT CAATGATATA TCGTATACTT CTCAATATTG     450
ATGCCAAAAA TGCTTTTTAT TTTGCCCAAA TTCTAAAGTC AGAGATGCTG     500
TCATAGAGTT ATGAGGAATT TATTCTTGGA ATTCCATCTT CTCTCACCCT     550
ATTCAATCAT AACCTCCAAT TTTTCAGTCA TTAAGAATCT CTTTTTGTAT     600
GACAAAGAAA ACCCGCAGCC GCTACCCTTT GGGTGCACAC AAGGCAAGTC     650
ATTAAGAATC TTTTATCTAG GAAGTAATAT TATACTCTTA TTAGCTCATG     700
GGTGATTGGC AACTAGTACA TGAAAGACAA AAAAACTGCC ATCAAGCAGG     750
AAACACTTTA ATATATAAAC ACTGTTCTCA AAACTAAAAT AATAATAAAT     800
AAAAACCCTT GAAAATGTTG AAGCCCTTAT TGCTTCATCA TTTTTGAGAA     850
TAGAAGCTTG AGAGTAGCAT CCTCCCTTAT TTGTTAAAGA ATGTAGGGAG     900
TTGGAAACAG TCTGAAGTGC TTGTGATACC ATCTGTAAGC ACTTGGATTC     950
AGGACATAAC AGAAATGGGT AAATTTTGAA AAGTAGCCAG TTTTTGCTCT    1000
GTTTGGTGTT TCATTGATCG AAGGATCATC ATATTTCAGT ACTGCAATAT    1050
GTTGGAAAGG TATTACTGTT TCATCTTTGA GGTTTGAGGC CATAGATTTA    1100
GTGCCCGTTG GATTAACTCA CTTTTAGGTG CTTTTGTCTT TTAAGCATTT    1150
TATAAGTTTT GGAGGTGTTT GGAAAGGTTA AAAAGTGCTT CTAAGCATTC    1200
ACTTTTTTGC CAAAAAAGTT TTAAAATAAG TCAAAAGTCG AATGTAGGGT    1250
ATCATCTACT TATGACTTTT AGCGTTTTGA CTTATAAATT ACTTTTATAA    1300
GCTCATCCAA ACAGGCCCTT GTTCATTTAT ACCTCCCTAA AGATCTTTGA    1350
CGAGACTGAA GGCTATTCTG CATATCGGTG TTGATAGTCT GAAGAAAATA    1400
ATCTGCCTAT ATACATCGAG CTTCTTTTCA ATTTATACAT CATTTAACAG    1450
AATTGCTAAT TAGTATCTTT AATTCTTTTT AATGACACTT AAAATGTTTT    1500
ACATCTTTTT ACTAATCTGA TTCTTAGTGG ACCCGTTGGA GATGGCGTGA    1550
TGGCTTCTGC AAGTGAAGTC AATGCCCTGA AAGATTCATT TTCAGCTTTA    1600
GATGTTGGTG TCGTGGCAGA TATCAAAACT GAAGACACTG TCAAGCAGAC    1650
TCCTGCTGTA GGCATCACAG ACTGCAAATG TGGGATGCCT TTGTGTATCT    1700
GCCAAGTGTC AGCTACACCA ACAACATCCA TTGCTTCACA GGAAAGGACA    1750
TATTTACTGG CCTTCAAATG CTTTTTGAGC AAATGACAAT TCCTTTATAT    1800
TTTTTTTTGA TTCCAGCAGG GAATTATTAT GCCAAATCCA ATTGTAAACA    1850
TAAATCCAAA ACCAAAAAAA AAAAAAAGTC TACAAGTCAC CACCACCACC    1900
AGTGAAGCCA TACCATCCTA CACCCGTATA CAAGTCTCCA CCACCACCAA    1950
CTCCCGTTTA CAAGTCACCA CCATCACCAG TGAAGCCATA TCATCCCTCA    2000
```

```
CCAACACCAT ACCACCCTAC ACCAGCATAC AAGTCTCCAC CACCACCAAC     2050
TCCAGTCTAC AAGTCTCCAC CACCAACCCA CTATGTTTCC TCCTCTCCCC     2100
CTCCTCCCTA CCATTACTAA GAAGTGAGTT TACTATATCT GAGGAAAAGC     2150
CTAATGTTGA GCTGAAAGAA AGGCATTTTC CATTTTCAAG AAGAAAATTA     2200
TAGTAAATAA TAAGGCTTAC AGAAGATCAG ACGAAGTTCT TTTGTAGCTT     2250
CATGTTATCT AACTAGTCTT AGTGATATAT TGTTTTTGTA CTCTATTTTT     2300
ATATATTACT TTTATGTGTC TTTGTGTATG TTTGCTCACT TTCAATCTTC     2350
TTGCAAAATG CAGAGATTAA TTATGAGATT ATCATGAATA AAATAAGTTA     2400
TTACTACTCC CATATGTTTT AAAAAAAAAA AAAACCATGG TACCCGGACC     2450
TCGAGGATCC GGGTACCATG GCACTGGCCG TCGTTTTACA ACGTCGTGAC     2500
TGGGAAAACC CTGGCGTTAC CCAACTTAAT CGCCTTGCAG CACATCCCCC     2550
TNNCGCCAGC TGGGCTAATA GCGAAGAGGC CCGCACCGAT CGCCCTTCCA     2600
ACAGTTGCGC                                                2610
```

SEQ ID NO. 6

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 1004 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato

PROPERTIES: Nematode-responsive cDNA
MISCELLANEOUS: cDNA designated as LEMMI 9

```
ACCATGATTA CNCCAAGCTT GCATGCCTGC AGGTCGACTC TAGAGGATCC      50
CCGGGTACCG AGCTCGAATT CGAGGATCCG GGTACCATGC CGGTATGGTA     100
CCCGGATCCT CGATTCGAGG ATCCGGGTAC CATGTTCGTT TAGCACAAAA     150
CAGGCATACT ATTCAATTCC CTTTCGTTCC AGAAGCATGG ATCTAATTGA     200
CAAGGCGAAG AATTTTGTGT CGGAGAAGAT AGCCAACATG GAGAAACCGG     250
AGGCAACCAT CACCGACGTC GATCTTAAGG GGATCGGTTT CGACGGCCTT     300
GCTTTTCACG CTAAAGTCTC CGTTAAGAAC CCTTACTCTG TTCCTATTCC     350
AATCATGGAG ATCGATTACG TCCTCAAAAG CGCCACCAGG GTAATCGCAT     400
CAGGAAGAAT TCCAGACCCA GGGAGCATAA AGGCAAATGA CTCAACCATG     450
TTAGATGTGC CAGTGAAGGT TCCTCACAGT GTGCTAGTGA GTTTGGTTAG     500
GGACATTGGA GGAGATTGGG ACGTCGATTA TACCCTGGAA TTGGGTCTCA     550
TTATTGATAT TCCGGTCATT GGCAACATCA CCATTCCCCT CTCTTATAGC     600
AGGCGAGTAT AAGCTTCCTA CATTGTCAGA TTTATGGAAG GGTGGAAAAG     650
AAGAAGAGAA AAAAGAAGAT GAAGAGGAGA AAGAAGATCC ATCAAAGGTT     700
GTTGAGATAT GAAGAGTTAT ACCTATCTAA TAATGTGGCT TTAATATGCC     750
TAGTTTCTCT TCTGTTGTTT TAATAACATA AAGTTTGTTT ACCTTATAAG     800
TATCATTTCA AGGATACAAA ATGCACAACT TTATGAAACT CACATTACTC     850
TTATCTCACC TGATTTGATG ATATGAAGAT TTGATGATGT TAGGTTTAAA     900
AAAAAAAAAA AAACCATGGT ACCCGGATCC TCGAATTCAC TGGCCGTCGT     950
TTTACAACGT CGTGACTGGG AAAACCCTNN NGTNAACCCA ACTTAATCGC    1000
CTTG                                                      1004
```

SEQ ID NO. 7

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 507 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato

PROPERTIES: Nematode-responsive cDNA
MISCELLANEOUS: cDNA designated as LEMMI 10

```
GGCCAGTGAA TTCGAGGATC CGGGTACATG GGATGAAAAA GGTGTGAAGA      50
AGGATAAGGA TAAGAAACCC AATAAGGAAA AGAAAGAGAA AAAGGATAAA     100
GGAAAGAAAG ATAAGAGCAA AGAGGAGTCG GAGGAAGAAG AGAAGGATGA     150
TGTAAAAGGG AAGAAGAAGG ATAAAGAGAA GAAAGATAAG AATAAAGAGT     200
TGTCGGAAGA AGAAGATAAT GAAGAGAAGG ATGATAAAGT AGGCAAGAAG     250
AAGGATAAAG AAAAGAAAGT CAAGGCGAAT GCGGCTGAAG TCGCCACAAG     300
AGAGCTAGAA GTTGAGGAAG ACAAGAAAGT ATCCGACGAT GAATCAGAAG     350
AGAAAAGTAA AAGCAAGCCA TGGTACCCGG ATCCTCGAAT TCGAGCTCGG     400
TACCCGGGGA TCCTCTAGAG TCGACCTGCA GGCATGCAAG CTTAANATAA     450
TCATGGTCAT AGCTGTTTCT GTGTGAAATT GTTATCNTCA CAATTCACAC     500
AACATAC                                                   507
```

SEQ ID NO. 8

SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 731 bp

STRANDEDNESS: double-stranded
TOPOLOGY: linear
MOLECULAR TYPE: cDNA to mRNA

ORIGINAL SOURCE: plant
ORGANISM: tomato

PROPERTIES: Nematode-responsive cDNA
MISCELLANEOUS: cDNA designated as LEMMI 11

```
CGGCCAGTGA ATTCGAGGNT NCGGGTACAT GGCCCCATCC TACAAGTTAC      50
CACCTCCACC AACTCCCATT TACAAGTCGC CACCACCACC AACTCCTGCC     100
TACAACTCTC CTCCACCACC TTACTATCTT TACACCTCTC CCCCTNNGGG     150
CTACCATTAC TAAAAAGCCA CTTCATTATA TCGAGGTAAT TAAGACAAAC     200
TAAAACTATT ACTAATGATG TGTCTAAAAT GCAAAGCAAT AAGTTGCTCA     250
ATTTCCTCAT GCAAATCATT AATATATAGA CCTATGCAGT CTATATTCTT     300
ATTTGGATAA CTTTAATTAC ATGTTCTCAT TCACAAAATT ATATTCTTAT     350
GCAGGAAAAG TAAAATGTTG AGCTTAAAGA AAGGCATTTT GCATTTTCGA     400
GAGACGATGA AAAAAGAAGA AAACTATAGT AAATAATAAG CCCCACATAA     450
GTCGAAGTTC TTTTGTAGCT TCATGTTATC TAAGCTAGTG ATATTGTTNG     500
TACTCTATTA TTTATATTTG TATTTTTACT TTTATGTCTT TCTGTATGTT     550
TGCTCAGTTT TAATATCCTA GCAAAATGCA AAGATTAATT ATGAGATGCA     600
TAAAATAAGT TATTACTATT AAAAAAAAAA AAAACCATGG TACCCGGAGG     650
ATCCGNNNNN NNTNGNGAGC TCGGTACCCG GGGATCCTCT AGAGTCGACC     700
TGCAGGCATG CAAGCTTGGC GTAATCATGG T                         731
```

CLAIMS

1. A nematode-responsive, preferably nematode-induced, plant promoter which can be isolated from genomic tomato DNA, upstream of a gene thereof having a DNA sequence which corresponds to a cDNA selected from the group consisting of SEQ ID nos. 1-8, preferably SEQ ID nos. 2-8, particularly SEQ ID nos. 4, 6 and 7, quite particularly SEQ ID no. 7.

2. A nematode-induced chimaeric gene, suitable for transforming a plant to protect it against nematode infection, which comprises the following operably linked, DNA sequences:

a nematode-induced promoter, preferably the nematode-induced promoter of claim 1, that is suitable to direct transcription of a foreign DNA at least substantially selectively, preferably selectively, in specific cells of the roots, preferably in the cells of fixed feeding sites, of the plant;

a first foreign DNA that encodes a first RNA and/or protein or polypeptide which, when produced or overproduced in the specific cells of the roots, preferably in the cells of the fixed feeding sites, of the plant, either a) kills, disables or repels the nematodes when the nematodes feed from the fixed feeding sites or b) kills the specific cells of the roots, preferably the cells of the fixed feeding sites, or a least disturbs significantly their metabolism, functioning and/or development, thereby at least

disturbing significantly, and preferably ending, the ability of the nematodes to feed from the fixed feeding sites of the plant; and
suitable 3′ transcription termination signals for expressing the first foreign DNA in the specific root cells, preferably the fixed feeding site cells.

3. The nematode-induced chimaeric gene of claim 2 wherein the nematode-induced promoter is suitable to control transcription of the foreign DNA at least substantially selectively in the cells of the fixed feeding sites of the plant.

4. A plant cell or plant cell culture transformed with the nematode-induced chimaeric gene of claim 2 or 3.

5. A plant or its seeds consisting essentially of the plant cells of claim 4.

6. The plant of claim 5 or its seeds, in which the nematode-induced promoter directs transcription of the first foreign DNA only substantially selectively in the specific root cells, preferably the fixed feeding site cells, of the plant and which also contains a restorer chimaeric gene, preferably in the same genetic locus as the nematode-induced chimaeric gene; the restorer chimaeric gene having the following, operably linked, DNA sequences:

a second promoter, such as the nematode-repressed promoter of claim 1, which can direct transcription of a second foreign DNA in cells of

the plant where the first foreign DNA is expressed, preferably substantially selectively in cells other than the specific root cells, preferably in cells other than the fixed feeding site cells, of the plant;

a second foreign DNA that encodes a second RNA and/or protein or polypeptide which, when produced or overproduced in cells of the plant, inhibits or inactivates the first foreign DNA or the first RNA or protein or polypeptide; and

suitable 3' transcription termination signals for expressing the second foreign DNA in plant cells.

7. A cell of the plant of claim 6 or a cell culture consisting essentially of the cells.

8. A process for rendering a plant resistant to nematodes, particularly sedentary endoparasitic nematodes, comprising the step of transforming the plant's nuclear genome with the nematode-induced chimaeric gene of claim 2 or 3 and optionally the restorer chimaeric gene of claim 6.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/01214

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/29; C12N15/82; C12N5/10; A01H5/00
A01N65/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H ; A01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	MOLECULAR & GENERAL GENETICS vol. 226, no. 3, May 1991, pages 361 - 366; GURR, S. J., ET AL.: 'Gene expression in nematode - infected plant roots' see the whole document ---	1-8
A	J. CELL. BIOCHEM. SUPPL. vol. 13D, 1989, page 323; NIEBEL, A., ET AL.: 'Molecular analysis of nematode induced giant cells in potato roots' * abstract M429 * --- -/--	1-8

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 SEPTEMBER 1992	29. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	PHYTOPHYLACTICA vol. 23, no. 2, 1991, MEETING HELD APRIL 7-10, 1991. page 182; DE WEALE, D.: 'Potential of plant genetic engineering for nematode control' * abstract *	1-8
A	EP,A,0 298 918 (CIBA-GEIGY) 11 January 1989 see column 6 - column 7	1-8
A	CHEMICAL ABSTRACTS, vol. 113, 1990, Columbus, Ohio, US; abstract no. 127723, JUN, W.: 'Preparation of transgenic plants for control of virosis' page 205 ; see abstract & CN,A,1 033 645 (FAMING ZHUANLI SHENQUING GONGKAI SHUOMINGSHU) 5 July 1989	1-8
P,A	WO,A,9 204 453 (UNIVERSITY OF LEEDS) 19 March 1992 see the whole document	1-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9201214 SA 60820

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0298918	11-01-89	AU-B- 620039	13-02-92
		JP-A- 1037294	07-02-89
CN-A-1033645		None	
WO-A-9204453	19-03-92	AU-A- 8502391	30-03-92